Effect of Non-Histone Chromosomal Proteins on Transcription *in vitro* in Sea-Urchin

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Non-histone chromosomal proteins prepared from chromosomal material of the sea-urchin *Paracentrotus lividus* affect RNA synthesis *in vitro*. 1. The extent of transcription can be radically changed from inhibition to stimulation, depending on the DNA/non-histone chromosomal proteins ratio. 2. A correlation exists between stage of development and influence on transcription. 3. Non-histone chromosomal proteins exert their action by intervening directly on some initiation step of RNA synthesis, as shown by the numbers of initiation events that take place in their presence or absence. 4. Stimulatory activity is observed only in restrictive conditions of ionic strength and temperature. These observations are in agreement with models that predict for non-histone chromosomal proteins a regulatory role on the transcription process exerted through a modulation of promoter availability.

It is generally postulated that RNA polymerase activity is regulated by the selective control of promoter availability (Travers, 1976). Basically the process should require first the recognition of a sequence and then the interaction with a binding region; RNA synthesis starting from an RNA-initiation point (Pribnow, 1975) would then follow. The transition from the recognition complex to the stable pre-initiation complex involves local 'melting' within the promoter (Saucier & Wang, 1972); agents that affect the stability of the double helix, such as ionic strength (Mangel & Chamberlin, 1974*b*) or organic solvents (Nakanishi *et al.*, 1974; Travers, 1974), effectively influence the transition necessary to initiate RNA synthesis. It has furthermore been observed that such transition occurs *in vitro* within a narrow range of temperature, as in the synthesis of *Escherichia coli* rRNA (Travers *et al.*, 1973) and in the transcription of phage- λ gal DNA and of phage- λ sex DNA (Nakanishi *et al.*, 1974). We have previously reported (Di Mauro *et al.*, 1977) that the initiation of RNA synthesis is also temperature-dependent for sea-urchin DNA, where one initiation event takes place every 2×10^4 base-pairs at 37°C and every 10^5 base-pairs at 12°C. Such temperature-dependent transitions were not observed for transcription of sea-urchin chromatin (Di Mauro *et al.*, 1977; Tsai *et al.*, 1976), where we found a 100-fold decrease in initiation events. This implies that some

factors other than RNA polymerase and template sequence intervene to mediate the formation of initiation complexes in eukaryotes.

In the present paper we report observations on the action of sea-urchin non-histone chromosomal proteins that lend support to current theories that predict a regulatory role for such proteins. They have a marked influence on template activity, show correlation with developmental stage and seem to abolish the temperature-dependent transitions observed on purified templates, but absent on chromatin.

Experimental

Materials

Nucleoside triphosphates were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. [3 H]UTP (14 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. *E. coli* B was grown in a complete medium (Burgess, 1969) to the late-exponential phase and kept as a frozen paste. Purified calf thymus histones (type II) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Spermatozoa from *Paracentrotus lividus* were obtained by two gastro-enteral injections of 0.55M-KCl. After thorough washing with sterile sea-water, spermatozoa were collected by centrifugation at 10000 rev./min in a SS34 Beckman rotor and kept frozen.

Fig. 1. Electrophoretic pattern (Panyim & Chalkley, 1969) of non-histone chromosomal proteins and other fractions at various steps of purification

For experimental details see under 'Methods'. 1, Calf thymus histones; 2, sea-urchin histones; 3, unextracted chromatin proteins; 4, chromatin proteins extracted by sonication in 1M-(NH₄)₂SO₄; 5, sea-urchin histone H₁; 6, sea-urchin non-histone chromosomal proteins.

Methods

Isolation of *E. coli* RNA polymerase, preparation of DNA and growth of sea-urchin embryos. These were done by procedures described by Di Mauro *et al.* (1977).

RNA synthesis. Conditions for RNA synthesis were as described by Di Mauro *et al.* (1977), both for reactions where reinitiation was inhibited and for normal synthesis. Incorporation without re-initiation was accomplished essentially by the procedure of Hymann & Davidson (1970), modified as described by Di Mauro *et al.* (1977).

Preparation of non-histone chromosomal proteins. Embryos were kept frozen at -12°C [freezing was done slowly in sea-water containing 10% (v/v) glycerol]. Thawed embryos were washed twice and resuspended in 3 vol. of buffer A [0.05M-Tris/HCl (pH 7.6)/1 mM-EDTA/10 mM-β-mercaptoethanol], and 0.25M-sucrose, homogenized in a loose Dounce homogenizer and centrifuged at 8000 rev./min in a Beckman SS34 rotor for 20 min. The pellet containing the nuclei was kept at 0°C for 10–20 min in buffer A containing 0.5% Triton X-100, washed twice in buffer A and resuspended in 60 ml of buffer A containing 1.6M-sucrose. It was then layered in six centrifuge tubes each containing 20 ml of buffer A containing 1.9M-sucrose and centrifuged at 22000 rev./min for 60 min in a Beckman SW 27.1 rotor. Purified nuclei were gently broken in a Dounce

homogenizer in hypo-osmotic medium [10 mM-Tris/HCl (pH 8.0)/5 mM-MgCl₂/5 mM-dithiothreitol containing 200 μg of phenylmethanesulphonyl fluoride/ml]; the resulting chromatin was washed twice and then resuspended in the same buffer made 1M with (NH₄)₂SO₄. The suspension was then sonicated six times for 20 s with 1 min cooling intervals [this was carried out with an MSE Sonifier (10 mm probe at 20000 Hz)] and centrifuged at 35000 rev./min for 2 h in a Beckman SW41 rotor. The supernatant containing the non-histone chromosomal proteins was made 65% saturated with (NH₄)₂SO₄ and centrifuged at 30000 rev./min for 1 h in a Beckman SW41 rotor. The resulting pellet was resuspended in 20 ml of 10 mM-Tris/HCl (pH 8.3)/5 mM-MgCl₂/1 mM-dithiothreitol containing 200 μg of phenylmethanesulphonyl fluoride/ml and extensively dialysed for 36 h against 6 litres (three changes) of the same buffer; contaminating lysine-rich histones are precipitated in this buffer and were removed by centrifugation (4 h in a Beckman SW41 rotor at 37000 rev./min, 4°C). The remaining clear supernatant, referred to as non-histone chromosomal proteins, was devoid of histones, as judged by electrophoresis (Panyim & Chalkley, 1969) (Fig. 1). The non-histone chromosomal protein solution was made 50% (v/v) in glycerol and kept at -20°C. In RNA-synthesis experiments non-histone chromosomal proteins were used as such.

The non-histone chromosomal protein fraction was also devoid of contaminating nuclease activity; this was shown as described by Di Mauro *et al.* (1972).

Results

Effect of non-histone chromosomal proteins on template activity

The effect of non-histone chromosomal proteins prepared from gastrula-stage embryos was tested in a transcriptional system composed of purified *P. lividus* DNA and *E. coli* RNA polymerase. Fig. 2(a) shows that the template activity of the DNA is affected by the presence of non-histone chromosomal proteins; in particular a normal template-saturation curve is observed in the absence of the proteins, but a sigmoid curve is obtained in their presence, especially at the lower concentration reported. A stimulation was also observed at higher concentrations of proteins, when a correspondingly larger amount of template was used. Stimulation was also observed for synthesis in conditions of no re-initiation (see below).

It seems therefore that alteration of template activity depends on the DNA/non-histone chromosomal proteins ratio; the same conclusion can be deduced from Fig. 2(b), where a fixed amount of proteins was tested at three different concentrations of template: oversaturating (50 μg/ml), saturating

Fig. 2. Template activity of sea-urchin DNA in the presence of non-histone chromosomal proteins from gastrulae (a) Increasing amounts of DNA were incubated (50 μl total vol.) for 5 min at 37°C in the absence (○) or in the presence of 0.1 (●), 0.4 (■) or 1.2 (▲) μg of non-histone chromosomal proteins, then 0.7 μg of *E. coli* polymerase and the synthesis mixture (see the Experimental section) were added to 100 μl final volume. Reaction was stopped after 15 min at 37°C. In both preincubation and synthesis 0.1 M-(NH_4)₂SO₄ was present. (b) Preincubation of non-histone chromosomal proteins with DNA and synthesis were as in (a). Preincubation of the indicated amount of non-histone chromosomal proteins was done in the presence of 0.8 (▲), 2.0 (○) and 5.0 (●) μg of sea-urchin DNA. Incorporation in the absence of non-histone chromosomal proteins was respectively 1750, 2100 and 2050 c.p.m. and was taken as 100% value.

(20 $\mu\text{g}/\text{ml}$) and undersaturating (8 $\mu\text{g}/\text{ml}$). The results indicate that a DNA/non-histone chromosomal proteins ratio optimal for stimulation is observed only at oversaturating concentrations of template and that a decrease in DNA leads to a decrease in the relative template activity. Variations of the amount of RNA polymerase (in conditions of template excess) did not alter the observed pattern (results not shown), showing that the enzyme concentration is not involved in the inhibition/stimulation effect, which seems to be determined by DNA/non-histone chromosomal proteins ratio only.

Correlation with the developmental stage

The effect of inhibition/stimulation of the template activity does not itself provide much information on the specificity attributes of the proteins under investigation. The sea-urchin system, however, offers the possibility of obtaining chromatin from embryos at different stages of development. If the proteins extracted had some physiological role in the control of transcription, one should observe a parallel *in vitro* with the RNA-synthetic activity *in vivo*. Fig.

3(a) shows the effect of non-histone chromosomal proteins purified from nuclei of freshly laid eggs, known to synthesize very little, if any, RNA [for a review of data on RNA synthesis *in vivo* during sea-urchin development see Giudice (1973)]. The effect of non-histone chromosomal proteins from eggs is only inhibitory, and a decrease in the amount of proteins used only leads to a decrease in the effect; we have never observed any appreciable stimulation by this material. Testing of the inhibitory capacity of the proteins extracted at different stages of development (Fig. 3b) shows that: (a) non-histone chromosomal proteins from eggs are able to block completely the transcription at a protein/DNA ratio of 15; (b) non-histone chromosomal proteins from embryos at higher stages of development have a decreased inhibitory capacity; (c) there is an increasing fraction of the template activity that cannot be blocked by very high concentrations of non-histone chromosomal proteins from developing embryos even at early stages. We have not observed, probably because of the limited sensitivity of the method, the same quantitative correlation in the stimulation effect.

Fig. 3. Effect of non-histone chromosomal proteins from various stages of development on transcription (a) Template activity of sea-urchin DNA in the presence of non-histone chromosomal proteins from mature eggs. Increasing amounts of DNA were incubated for 5 min at 37°C in the absence (○) or in the presence of 0.05 (●) or 1.0 (■) μg of non-histone chromosomal proteins. Conditions were as in Fig. 2(a). (b) Differential inhibitory activity of non-histone chromosomal proteins purified from embryos at different stages of development. The effect of the amount of proteins shown in abscissa was tested, as above, on 0.8 μg of sea-urchin DNA (subsaturating amount). Non-histone chromosomal proteins were from mature eggs (●), morulae (32 cells) (○), hatching blastulae (Δ) or mid-gastrulae (□). The 100% value (in the absence of non-histone chromosomal proteins) was 1850 c.p.m.

Mode of action

(a) *Frequency of initiation sites.* The natural target for regulatory proteins of the chromatin is one (or more) of the steps that lead to initiation of the synthesis of RNA. The fact (see Fig. 2a) that the amount of non-histone chromosomal proteins changes the saturation point of a DNA-saturation curve is a strong indication that their positive and negative activity is exerted on one of the initiation steps. At the optimum DNA/non-histone chromosomal proteins ratio, for instance, the DNA shows a 2-fold increase in transcriptional capacity. By directly calculating how many initiation sites are available, it can be shown that such an increase is due to an effective increase of availability of initiation sites. This can be conveniently achieved by the Hymann-Davidson technique (Hymann & Davidson, 1970) referred to in the Experimental section: the enzyme was preincubated with template and proteins (as indicated in the legend of Table 1) in a reaction mixture lacking one nucleoside triphosphate, in ionic conditions that allowed initiation and start of the synthesis, but not extensive elongation, to occur; after 15 min the missing triphosphate was added and ionic strength was increased to 0.4M $(\text{NH}_4)_2\text{SO}_4$, thus allowing propagation, but no new

initiation, to take place. The determination of incorporated radioactivity, the analysis of the length of RNA chains produced and the calculation of the initiation sites was performed as described by Di Mauro *et al.* (1977).

Table 1 reports the results of the calculation of available initiation sites in relation to the state of the template used: a decrease in the amount of RNA synthesis corresponds to a decrease in the frequency of initiation sites and vice versa. This evidence, taken together with the displacement of the DNA-saturation point, shows that non-histone chromosomal proteins act by altering the ability of the template to support synthesis.

(b) *Conditions that alter template activity of DNA.* It is known that factors that induce alteration of the conformation of the template [supercoiling (Hayashi & Hayashi, 1971), glycerol and other organic compounds (Nakanishi *et al.*, 1974; Travers, 1974), ionic strength (Mangel & Chamberlin, 1974b) and temperature (Travers *et al.*, 1973; Mangel & Chamberlin, 1974c)] have a strong influence on its template activity.

(i) *Ionic strength.* It is known that increased ionic strength affects enzyme conformation, the stability of binding to the DNA template and the rate and

Table 1. Influence of non-histone chromosomal proteins on the number of initiation sites

RNA polymerase (7.0μg) was used in a transcriptive system containing 10μg (80% of saturation) or 60μg (3-fold excess) of sea-urchin DNA. The conditions of synthesis and of calculation of the initiation sites are reported in the text. The amounts, of egg and gastrula non-histone chromosomal proteins used were 1.0μg with limiting DNA and 10μg of both non-histone chromosomal proteins with excess of DNA. The average chain length (±10%) of the RNA chains produced was 390 nucleotides in the limiting DNA condition, and 480 nucleotides for the excess DNA condition.

Relative DNA saturation	Transcription efficiency (%)		10 ⁻⁴ × No. of base-pairs/initiation site	
	80%	3-fold excess	80%	3-fold excess
No addition	100	100	2.8	12.0
Egg non-histone chromosomal proteins	32	65	7.8	19.0
Gastrula non-histone chromosomal proteins	125	80	2.0	14.0

Fig. 4. Influence of ionic strength on effect of non-histone chromosomal proteins

In (a) and (b) template activity of sea-urchin DNA was measured in the presence of 0.005 (○) or 0.1 (●) M-(NH₄)₂SO₄. Assay conditions were as in Fig. 2(a). (a) Non-histone chromosomal proteins absent; (b) 0.2μg of gastrula non-histone chromosomal proteins present. (c) Influence of (NH₄)₂SO₄ concentration on transcription in the absence (○) or presence (●) of 0.2μg of gastrula non-histone chromosomal proteins. Assay conditions were as in Fig. 2(a); 0.7μg of RNA polymerase and 1.0μg of sea-urchin DNA were present per assay.

duration of RNA-chain elongation (for a review see Chamberlin, 1974). It has in particular been shown that increasing salt concentrations progressively decrease the fraction of enzyme molecules that can initiate an RNA chain (Mangel & Chamberlin, 1974b), and evidence has been presented that low ionic strength favours the formation of a rapid-starting binary complex between DNA and RNA polymerase, whereas a non-starting or slow-starting complex is favoured at high ionic strength. In the *E. coli* enzyme-*P. lividus* template system, we can observe that a three-fold increase in the amount of DNA necessary to saturate a given amount of

enzyme occurs when the (NH₄)₂SO₄ concentration increases from 0.005 M to 0.1 M, i.e. the availability of the template to the enzyme is decreased at higher ionic strength (Fig. 4a). The presence of gastrula non-histone chromosomal proteins has no effect at low ionic strength, where DNA displays more initiation sites to the enzyme, but markedly changes the template activity at 0.1 M-(NH₄)₂SO₄: non-histone chromosomal proteins efficiently antagonize the decrease of template availability caused by increased ionic concentrations.

Taking into account the evidence obtained by Mangel & Chamberlin (1974b) that the lower amount

Fig. 5. Influence of non-histone chromosomal proteins on the temperature-dependent availability of sea-urchin template

Reactions were formed in conditions of no re-initiation (see the text). Preincubation of RNA polymerase (0.7 μg) and sea-urchin DNA was done at 0°C, in the absence (○) or presence (●) of 0.1 μg of gastrula non-histone chromosomal proteins. Elongation occurred at 37°C.

of initiation events at high salt concentration is due to a shift of equilibrium between non- or slow-starting and rapid-starting complexes in favour of the former, the experiment reported here is interpreted to mean that non-histone chromosomal proteins favour the formation of stable rapid-starting complexes; in low salt concentrations synthesis starts with an already high efficiency and the effect of non-histone chromosomal proteins cannot be seen.

(ii) Temperature. RNA synthesis *in vitro* is strongly affected by temperature. Essentially every step of the synthesis is influenced, from modification of the structure of the DNA itself (Von Hippel & McGhee, 1972) to the rate of RNA-chain elongation (Manor *et al.*, 1969). The most dramatic effect has, however, been observed (Mangel & Chamberlin, 1974c) on the formation of the binary complex between RNA polymerase and DNA, which is able to start synthesis; it was therefore decided to test in this respect the influence of the sea-urchin non-histone chromosomal proteins in the transcription of the homologous template.

To study the initiation step separately from subsequent synthetic stages we made use of the technique described by Di Mauro *et al.* (1977) that allows separation of initiation from elongation. Fig. 5 shows how template efficiency of sea-urchin DNA is affected by the temperature. The initiation step was allowed to occur at 0°C, the elongation at 37°C. In the absence of non-histone chromosomal proteins the amount of DNA necessary to saturate 0.7 μg of

the enzyme at 0°C is 7 μg, a value 5 times that necessary to saturate the same amount of enzyme at 37°C (see Fig. 1a). This effect of temperature on the initiation step is generally well known, and we have described it for sea-urchin DNA (Di Mauro *et al.*, 1977). Fig. 5 also reports the influence of non-histone chromosomal proteins on the template capacity at 0°C. The effect is clear-cut, and shows that in the presence of non-histone chromosomal proteins (a) DNA is more available, and (b) the saturation obtained at 0°C is identical with that obtained at 37°C in the presence of proteins (see Fig. 2a) (in the presence of non-histone chromosomal proteins the saturation occurs at 1 μg at both 0 and 37°C; in their absence it occurs at 2 μg at 37°C, or at 7 μg at 0°C). An increase in temperature thus increases the initiation sites available; at 0°C non-histone chromosomal proteins mimic the temperature effect, giving the DNA at 0°C a transcriptional capacity even higher than that shown at 37°C by the DNA alone.

This result is in agreement with that of the ionic-strength experiment: the action of the non-histone chromosomal proteins is mostly evident in conditions (high salt or low temperature) that disfavour rapid starting-complex formation.

Discussion

Current theories on the regulatory function of the chromatin imply a fundamental role of non-histone chromosomal proteins (Paul, 1972). Evidence is rapidly accumulating that a specific regulatory role can also be maintained by these proteins *in vitro* (Gilmour & Paul, 1973; Haxel *et al.*, 1973; Barret *et al.*, 1974), in different transcriptive systems: chromatin is not after all a particularly delicate material. RNA polymerase activity is thought to be regulated by the selective control of promoter availability, and much direct evidence for this has accumulated in prokaryotes (Travers, 1976); in eukaryotes such a selective control is supposed to be basically of the same nature, although much more complex because of the higher integrative necessities of the eukaryotes (Britten & Davidson, 1969).

The present paper describes some general properties of the sea-urchin non-histone chromosomal proteins and tests whether their function can be correlated with promoter availability.

The developing sea-urchin embryo is an appropriate system for this kind of study, because it offers a well-synchronized material for the observation of stage-related differences of the chromatin components and because of a programmed RNA synthesis [i.e. rRNA is not synthesized before the gastrula stage and histone mRNA is essentially the only product during the first few cleavage divisions; for a review of this subject see Giudice (1973)].

The experiments reported in which a specificity of

the chromatin function was shown made use of *E. coli* RNA polymerase and not of the corresponding homologous multiple forms, showing that basic specificity resides on the template and not on the enzymic system (thought to provide only a gross control). Moreover, detailed knowledge of the various steps of the *E. coli* RNA polymerase-template interaction is available. In this respect particularly useful is the demonstration (Mangel & Chamberlin, 1974a) that the discriminatory factor in initiation is the existence of an equilibrium between two forms of binary complex, the slowly or non-initiating and the rapidly starting, and that ionic strength and/or temperature affect the synthesis of RNA by influencing this equilibrium (Mangel & Chamberlin, 1974b,c).

One basic characteristic of the non-histone chromosomal proteins that we have purified from developing sea-urchin embryos is that their stimulatory-inhibitory action is a function of the amount of the template present (that is of the relative proportions enzyme/template/non-histone chromosomal proteins).

Many examples of more or less purified protein factors that stimulate RNA synthesis *in vitro* have been reported (Stein & Hausen, 1970; Jacquet *et al.*, 1971; Di Mauro *et al.*, 1972; Lee & Dahmus, 1973; Seifart *et al.*, 1973; Sudgen & Keller, 1973; Teissere *et al.*, 1975). Some exhibit pronounced enzyme specificity and have much greater stimulatory effect on eukaryotic RNA polymerase form B (Stein & Hausen, 1970; Seifart *et al.*, 1973; Sudgen & Keller, 1973). Other factors, such as π -factor purified from *Saccharomyces cerevisiae* (Di Mauro *et al.*, 1972), seem to stimulate some step of the transcription process that is common to a large number of enzyme-template combinations; we have in addition observed that π -factor has a stimulatory-inhibitory action that depends, as for sea-urchin non-histone chromosomal proteins, on the amount of template present (Di Mauro *et al.*, 1974). A correlation of the stimulatory activity with the excess of template has been reported also for the *E. coli* factor H (Jacquet *et al.*, 1971). The widespread occurrence (*E. coli*, yeast, sea urchin) of this effect leads one to think that some general aspect of the initiation of transcription *in vitro* is affected by this type of protein [defined as type-II factors (Lukacs & Stein, 1976)]. The observation also that spermine, spermidine, polylysine or a mixture of histones (Hall *et al.*, 1973) in conditions of enzyme shortage, or histone I alone (Lukacs & Stein, 1976; Konishi & Koide, 1971), in the appropriate range of concentration, have a dose-dependent effect on the amount of RNA synthesis, further generalizes the interest of the system and indicates that it may be possible to construct simple model systems.

Besides these general aspects correlated with the

mode of action, it is noteworthy that sea-urchin non-histone chromosomal proteins are also endowed *in vitro* with some specificity, as shown by the lack of stimulation by non-histone chromosomal proteins prepared from eggs and by an inhibitory power differentially correlated with the synthetic capacity *in vivo*. It will be of interest to test the populations of RNA synthesized *in vitro* for similarities with those made *in vivo*. Such observations of relative specificity, although only quantitative and therefore not completely satisfactory, are, however, sufficient to rule out the most trivial explanations and indicate a physiological role. We suggest that such a role is of the kind described for non-histone chromosomal proteins in the model proposed by Paul (1972).

The evidence that supports such a hypothesis is: (a) the action of the proteins, being a function of the DNA/non-histone chromosomal proteins ratio, is exerted directly on the DNA; (b) their influence is exerted directly through initiation; (c) the conditions that shift the equilibrium from rapid-starting complex to non- or slowly-starting complex, dis-favouring the productive synthetic events, are counter-balanced by non-histone chromosomal proteins; noteworthy is the removal of the temperature-dependent transitions normally exhibited by the purified template.

The profound effect exerted by sea-urchin non-histone chromosomal proteins on the template activity of the DNA seems to render it more similar to the chromatin state. This consideration may help to understand the observation that at high protein/DNA ratio the promoter enhancement is lost and substituted by inhibition; considering that the main function of chromatin proteins should be the imposition of specific limitations on the sequences transcribed, at high protein/DNA ratio this limitative function may become the quantitatively more important one. Another explanation of the observed inhibition at high protein/DNA ratio might be that enhancement and inhibition are each due to different, unequally active, protein subpopulations. The results presented here, however, are not sufficient to allow us to speculate more on this point: the possibility of obtaining purified ribosomal (*E. di Mauro & M. Pomponi*, unpublished work) and histone (Birnstiel *et al.*, 1974) genes in this material will provide the means of studying more precisely the function of non-histone proteins.

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